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WESTERN BLOT ASSAY OF SELECTED PATIENTS BLOOD INFECED WITH HIV : IN AND AROUND SALEM DISTRICT, TAMILNADU, INDIA.

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ABSTRACT

The human immunodeficiency virus (HIV) is the etiological agent of acquired immunodeficiency syndrome and demonstraction of an antibody response specifically directed against HIV proteins is accepted as evidence of infection. Westernblot is the most commonly used test to conform Ahiv-I infection to perform the westerNblot the HIV-I antigens are separated on the basis of their molecular weights and antibodies to each components aare detected as distint bands. The following HIV-I antigens are currently identified in the westenblot ENVELOP(gp160, gp120,gp41)GROUP ANTIGENS (p55,p40,p24,p18)and POLYMERASE(p51,p65 andp31). To determine the indeterminate HIV-I and HIV-2 western blot results of HIV infected individuals. The westernblot is the confirmatory test for HIV infection. The infected blood samples had tested should have atleast one HIV characteristic band and any one oh HIV-2 band. We obtained information regarding HIV risk factor and peripheral blood specimen for following test , HIV-TRI-DOT for detection of antibodies in the blood samples.

KEYWORDS: HIV-1 & 2, Western Blot, Antigens (GAG, ENV, POL)

INTRODUCTION

Human Immunodeficiency Virus (HIV) is the etiological agent of Acquired Immunideficiency Syndrome (AIDS). HIV infection is now recognized worldwide as a major public health. Hence screening methods play an important role in disease detection. The most common immune assay used for the detection of antibodies to HIV-I AND HIV-2 are for the Enzyme – Linked Immunosorbent Assay (ELISA), rapid tests and the immunoblot or Western blot assay which are easy to perform. (Chattopathyay *et.al*. 1996)

The Western blot test can be used as a more specific and supplemental assay on human serum or plasma. Specimen found repeatedly reactive using ELISA. The HIV-i viral antigens are separated by gel electrophoresis, electrically transferred to nitrocellulose membrane strip, which is impregnated with a specific HIV -2 antigen band. Each strip also has an internal serum inbuilt quality control band. (Towbin *et.al*. 1979 and Gallo 1987)

In individuals infected with HIV, antigens appears first before anti HIV but due to seroconversion the antigen is lost and antibody develops within 1-2 months after infection and therapy by the level of the antibody increase . However p24 antibodies level decrease with time in advance stage of infection as shown in the graph. Hence, in advance stages of the infection , the p 24 band on the HIV westernblot strip may either be light or absent (Tedder *et.al*. 1988).

THE PRINCIPLE OF THE WESTERN BLOT

The HIV westernblot is manufactured from HIV-1 cell line . The HIV –I viral aantigen is purified and then separated by SDS gel electrophoresis . SDS denati\ures viral components and yield proteins which migrate in the gel according to their molecular weight to produce various bands . Low molecular weight component migrate faster and are found at the bottom of the gel. While high molecular weight proteins remain near the as top .They are then transferred from SDS-PAGE gel on to nitrocellulose membrane is cut and packaged strips.

To perform the assay, the strip is incubated with the patient serum/ plasma diluted in a buffer. Antibodies to HIV-1and HIV-2 if present, bind to viral antigens located on the strip. Unbound material is washed off and then the strip is incubated with anti- human IgG conjugated to alkaline phosphate.

After washing the unbound conjugate, substrate is added which results in the staining of bands, if antibodies of HIV-1 antigens are present in the sera any two ENVELOPE and more of the following bands will be seen. P24,p31,gp41,p51/p55.p66.gp120and gp160. If antibodies to HIV -2 antigens are present, HIV-2 band is also observed along with some of the other bands. If HIV specific antibodies are not present, the band pattern

does not meet the required criteria (Aldovini and Walker1990).

MATERIALS AND METHODS

The infected blood samples were collected and allow for clotting and then 1mln of serum can be separated. The serum sample were cold stored and then taken for assay. The samples from about 100 patients were collected and the details of the patients are kept secret because of ethical consideration. For, this research about 165samples were collected and within that samples 100 were reactive in the western blot assay. The study was carried out from June2007-December 2008, during this 18 months several samples were taken and finally this result is found.

SPECIMEN COLLECTION AND PREPARATION

Collect blood in a clean dry sterilized vial and allow it to clot. The serum was separated by centrifugation at room temperature. It is recommended that fresh samples should be used. If serum is not be assayed immediately, it should be stored at 2-8°C or frozen at -20°C. Serum may be stored at 2-8°C for upto 3 days and stored frozen at -20°C for 3 months (Towbin *et.al*. 1979).

ASSAY PROCEDURE – RAPID ASSAY:

The test kit was brought up the room temperature and the samples were brought up to room temperature (25-30°C) before use and all incubations are to be carried out on a Western Blot / Rotary Shaker (60-70 rpm) at room temperature.

Required number of strips and trays are taken from thefrom the kit. Place one strip in each tray with numbered side up. Note down the strip number with respect to samples and control on the worksheet for correct identification. Always include strips for positive and negative controls with each run.Working wash buffer i sprepared s according to the number of tests to be run.

Add 2ml of working wash buffer to each tray and incubate the strips for atleast 5 minutes at room temperature. Remove buffer by aspiration. Prepare Working diluents buffer is prepare d according to the number of tests to be run.

Add 2ml of working diluents buffer to each tray. Add 20µl of patient sera and controls to appropriate wells.

The trays were covered and incubated for 1 hour at room temperature (25-30°C) on a Western Blot shaker. The care should taken to mark the cover also to prevent interchange of covers which may lead to cross contamination. Carefully remove covers, aspirate solution completely from tray and discard into sodium / calcium hypochlorite solution.

The strip were washed with 2ml working wash buffer 3 times for 5 minutes each with shaking.

The working conjugate solution is prepared according to the number of tests to be run. Add 2ml of working conjugate solution to each tray. Cover tray with corresponding cover and incubate on Western Blot shaker for 1 hour. Never interchange the cover of trays to avoid contamination.

Aspirate conjugate, wash each strip with 2ml working wash buffer 4 times for 5 minutes each with shaking aspirate wash solution completely from the tray at the end of the last washing.

2ml of substrate is added to the solution of each tray. Cover tray and incubate for 0.5-15 minutes away from the light preferably in dark till bands develop. Make a careful decision to decide the time of incubation from 0.5 to 15 minutes. Continue to observe the reaction till gp160/gp120/gp41 appear and stop the reaction after their appearances so as to avoid excessive background making the observation difficult.

However, in case the above bands do not appear, then continue the reaction upto the point

Before strong background is formed on the strip up to 15 minutes, whichever is earlier.

Aspirate substrate, add distilled water and wash strips to stop the reaction. Remove the strips on paper towels and amount on worksheet keeping numbered side up. Observe band pattern and grade the results. For storage keep strips in dark.

RESULTS

The presence or absence of antibodes to HIV-1, in a serum sample is determined by comparing each strip

Description of bands observed on strip reacted with positive control

Molecular Wt.(kDa)	Gene	Antigen	Description
gp160	ENV	Polymeric form of gp41	Broad diffused band
gp120	ENV	Outer membrane	
p66	POL	Reverse Transcriptase	Discreet band
p55	GAG	Precursor protein	Fused Spread band, Single band
p51	POL	Reverse Transcriptase	
gp41	ENV	Transmembrane	Appears as 2-3 different bands, diffused band
p31	POL	Endonuclease	Single band
p24	GAG	Core Protein	Broad band
p17	GAG	Core Protein	Broad band

Fig. 1. HIV postive samples shows hiv genes presence

Hiv negative sample and control sample wich are not showing hiv genes

PRESENCE OF VARIOUS ANTIGENS IN THE INFECTED SAMPLES

Sample No	Core Antigens (GAG)			Endonuclease Polymerase Antigens (POL)			Envelope Antigens (ENV)			Serum Control Band	HIV-2 Specific Band
	p^{17}	p^{24}	p^{55}	p^{31}	p^{51}	p^{66}	gp^{41}	gp^{120}	gp^{160}		
1	—	P	—	—	—	P	—	P	—	P	—
2	—	P	—	—	—	—	P	—	P	P	—
3	—	—	P	—	—	P	—	P	P	P	—
4	—	P	—	—	P	—	—	P	P	P	—
5	—	—	—	—	—	—	P		P	P	—
6	—	—	—	—	—	—	—	P	P	P	P
7	P	—	—	—	P	—	P	—	—	P	—
8	—	P	—	—	—	P	—	P	—	P	—
9	P	—	—	P	—	—	P	—	—	P	—
10	—	—	P	—	P	—	—	P	—	P	—
11	—	P	—	—	—	P	P	—	P	P	—
12	—	P	—	—	P	—	—	P	—	P	—
13	—	—	P	—	—	P	P	P	—	P	—
14	—	P	—	—	P	—	—	—	P	P	—
15	—	P	P	—	—	P	P	P	—	P	—
16	—	P	—	—	—	P	P	—	P	P	—
17	P	—	—	—	—	P	P	—	—	P	—
18	P	—	—	P	—	—	—	P	P	P	—
19	—	—	P	—	—	P	—	P	P	P	—
20	P	—	—	P	—	—	P	P	—	P	—
21	—	—	—	—	—	—	—	P	P	P	P
22	—	P	—	P	—	—	P	P	—	P	—
23	P	—	—	—	P	—	P	P	—	P	—
24	—	—	P	—	P	—	—	P	P	P	--
25	—	P	—	—	P	—	—	P	P	P	—
26	P	—	—	P	—	—	P	P		P	—
27	P	—	P	—	P	—	—	P	P	P	—
28	—	P	—	P	—	—	—	P	P	P	—
29	P	—	—	P	—	—	—	P	P	P	—
30	—	—	P	—	—	P	—	P	P	P	—
31	—	P	P	—	—	P	P	P	—	P	—
32	—	—	P	—	P	—	—	P	P	P	—
33	—	P	—	P	—	—	P	P	—	P	—
34	—	—	P	—	P	—	—	P	P	P	—
35	—	P	—	—	P	P	—	P	P	P	—
36	—	P	—	—	—	P	—	P	P	P	—
37	—	—	P	—	—	P	—	P	P	P	—
38	—	—	P	—	P	—	P	P	—	P	—
39	—	P	—	—	P	—	—	P	P	P	—
40	—	P	—	—	—	—	P	—	P	P	—

41	—	—	—	—	P	—	—	P	P	P	—
42	—	—	—	P	—	—	—	P	P	P	—
43	P	—	—	—	—	—	P	P	—	P	—
44	—	—	P	—	—	—	—	P	P	P	—
45	—	—	P	—	—	P	—	P	P	P	—
46	P	—	—	—	—	—	P	P	P	P	P
47	—	—	P	—	P	—	—	P	P	P	—
48	—	P	—	—	—	—	P	P	—	P	--
49	—	P	—	—	—	P	—	P	P	P	—
50	—	—	P	—	—	P	—	—	P	P	—
51	—	—	P	—	—	P	—	P	P	P	—
52	—	—	P	—	P	—	P	P	—	P	—
53	P	—	—	—	P	—	P	P	—	P	—
54	—	—	P	—	—	—	—	P	P	P	—
55	—	—	—	—	—	—	—	P	P	P	P
56	—	—	P	—	—	—	—	P	P	P	—
57	—	P	—	—	—	—	—	P	P	P	—
58	—	P	—	—	—	—	P	P	—	P	—
59	—	P	—	—	P	—	P	P	—	P	—
60	—	—	P	—	—	P	—	—	P	P	—
61	—	P	—	—	P	—	—	P	P	P	—
62	P	—	—	P	P	—	P	—	P	P	—
63	—	P	—	—	P	—	P	P	—	P	—
64	P	—	—	P	—	—	—	P	P	P	—
65	—	—	—	—	P	—	—	P	P	P	—
66	—	P	—	—	—	—	—	P	P	P	—
67	—	P	—	—	P	P	—	P	—	P	—
68	—	—	P	—	—	—	P	—	P	P	—
69	P	—	—	—	P	—	—	P	P	P	—
70	—	—	—	—	—	—	—	P	P	P	P
71	P	—	—	P	—	—	P	P	—	P	—
72	—	P	—	—	—	P	—	P	—	P	—
73	—	—	P	—	—	—	—	P	P	P	—
74	P	—	—	P	—	—	P	P	—	P	—
75	—	P	—	—	—	P	—	P	P	P	—
76	—	P	—	—	P	—	P	P	—	P	—
77	—	—	P	—	—	P	—	P	P	P	—
78	—	P	—	—	—	—	—	P	P	P	—
79	—	—	P	—	—	P	—	P	P	P	—
80	P	—	P	—	—	P	—	P	P	P	P
81	—	P	—	—	P	—	P	P	—	P	—
82	—	P	—	P	—	—	P	P	—	P	—
83	—	P	—	—	P	—	—	P	P	P	—
84	—	—	P	—	—	—	—	P	P	P	—
85	—	—	P	—	—	—	—	P	—	P	—
86	—	P	—	—	—	P	P	P		P	—
87	—	—	P	—	—	P	—	P	P	P	—
88	—	—	P	—	P	--	—	P	P	P	—
89	—	P	—	—	P	—	—		P	P	—
90	P	—	—	P	P	—	—	P		P	—
91	—	—	P	—	—	P	—	P	P	P	—
92	—	P	—	—	—	P	—	P	P	P	—
93	P		—	P	P		P	P	—	P	—
94	—	P	—	—	P	P	P	—	—	P	—
95	—	—	—	—	—	P	—	P	P	P	P
96	—	—	P	—	—	P	—	P	P	P	—
97	—	P	—	—	P	—	P	P	—	P	—
98	—	P	—	P	—	P	P	P	—	P	—
99	P	—	—	P	—	—	P	P	—	P	—
100	—	—	P	—	—	P	—	P	P	P	—

with the negative and positive control strips. The description of the various bands is given below –

DISCUSSION

HIV/AIDS, a global problem which leads to the death of several million people, causes severe effects throughout the world. Even medical advances that have produced number of drugs that are safe and effective against the virus, are still a major cause of death; disability and social and economical upheaval for millions of people around the world. The HIV belongs to two categories namely, HIV-1 and HIV-2. These two types are mostly found

in the blood samples of the infected patients.Since then, HIV-1 has spread rapidly throughout the country and has been reported in every major city. Today , India has the second largest number of HIV-1infected individuals in the world , next to South-Africa (UNAIDS and WHO2004)Genetic analysis of HIV-1 circulating in different parts of India, the phenotypic and genotypic analysis of HIV-1 in India indicates that they are isolated from late stage of infection.(Sathiyavathi *et al*.,2007)

There is also a high molecular weight non –specific band above 160 is presumed to be a GAG-POL precursor protein. Antibodies of p24 are in excess during the early stage and Antibody level decress with the advance stage of infection , Antibodies of p31 could be low due to low immunity HIV -2 Band is indicative and intensity will be less than serum controle. The western blot is the confirmatory test for HIV infection ,here we found 100 patients were positive and reactive in the assay,hence we continue the the samples for further research.

Generally HIV-1 are the initial stage of the infection that are of early 5 years and the presence of HIV-2 indicates that are well developed stage of the infection. But when both HIV-1 and HIV-2 presence indicates that the infection is of last stage in the patients, that a period preface to the death. gp41, C terminal of gp120 and gp36 representing the immunodominant regions of HIV-1 and HIV-2 envelope gene structure.

An enzyme linked immune assay(ELISA) using recombinant proteins corresponding to large segments of the immunodeficiency virus gag ,pol, and env gene products was developed to conform the presence of antibodies to HIV in sera reactive whole cell derived virion screening ELISAs .Serum samples from HIV-infected individuals (asymptomatic,acquired immune deficiency syndrome(AIDS) related complex and AIDS). A positive reaction was defined as reactivity against an env and least one other (either gag or pol)HIV gene product negative was defined as no reaction with antigens and indeterminate was defined as reactivity with gag or pol (or both) or with env alone. To date , the only routinely used method for demonstrating the presence of hiv in vivo are enzyme linked immune sorbent assay .In western blot the HIV proteins are dissociated and placed on a poly acrylamide gel slab.After electrophoresis they are transferred to a nitrocellulose membrane by electroblotting . The patients serum is added to the antigen preparation . The immunogenic HIV proteins are thought to be encoded by three genes namely gag, pol and env. The gag gene encodes a precursor p53/55, which is then cleaved to p24/25 and the env genes encodes the precursor protein p160 which is cleaved to p120and p41/45.We have collected 165 serum samples and among them 230were indeterminate ,45 were negative and 100 samples found positive –that infected with HIV infection . In the100 samples only 6 samples found HIV-2 and 94 were HIV-2.

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